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INTERNATIONAL APPLICATION PUBLISHED

WO 9603139A1

(51) International Patent Classification 6 : <b>A61K 38/00, 38/16, 31/79, 31/195, 31/13, 31/16</b>		<b>A1</b>	(43) International Publication Date: 8 February 1996 (08.02.96)
(21) International Application Number: PCT/US95/09287		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 20 July 1995 (20.07.95)		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 08/281,427 27 July 1994 (27.07.94) US			
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(54) Title: USE OF NITRIC OXIDE OR NITRIC OXIDE ADDUCTS TO PRESERVE PLATELETS			
(57) Abstract  A method of preserving platelets comprising contacting platelets with an effective amount of nitric oxide, a compound which releases, delivers, or transfers nitric oxide, or cGMP or a derivative or analogue thereof. Such compounds inhibit platelet activation. By inhibiting platelet activation, one is able to store platelets for longer periods of time, and improve the quality of platelets used in transfusions.			

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**Use of Nitric Oxide or Nitric Oxide Adducts  
to Preserve Platelets**

This invention relates to preserving platelets. More particularly, this invention relates to preserving platelets by contacting platelets in vitro with nitric oxide, or a compound which is capable of donating, releasing, or transferring nitric oxide.

Platelet transfusions are used by physicians and hospitals for a large number of uses, such as, for example, the treatment of thrombocytopenia. There are two major types of platelet products used in blood banks, platelet concentrates and pheresis platelets (also known as single donor platelets).

Platelet concentrates are prepared from units of red blood cells. In general, from 4 to 10 platelet concentrates are pooled together for administration to patients. Two centrifugation steps are used in platelet concentrate preparation. In the first step, whole blood (in an amount of about 450 ml  $\pm$  45 ml) is subjected to a "light spin" (e.g., about 2,000 xg for 5 minutes), after which a supernatant fluid, sometimes referred to as platelet-rich plasma, is removed. This supernatant then is subjected to a "heavy spin" (e.g., about 5,000 xg for 5 minutes) which pellets the

platelets. The plasma then is discarded, except for 30-50 ml, which contain the platelets. The platelet bag is left stationary for a period of time of about 1 hour, and the platelets then are resuspended, either by manual manipulation or by placing the bag on a rotator for about 2 hours. Activation of platelets (as measured by expression of P-selectin (GMP-140 or CD62) has been noted immediately after collection and after blood bank storage. (Fijnheer, et al., Transfusion, Vol. 30, pgs. 634-638 (1990); Rinder et al., Transfusion, Vol. 31, pgs. 409-414 (1991)).

Single donor platelets are collected from individuals whose blood is subjected to platelet pheresis. The blood is passed through a revolving bowl, and only platelet-rich plasma is removed from the donor. Activation of platelets (as measured by expression of P-selectin (GMP-140 or CD62) has been noted in the products collected by this method (Triulzi, et al., Transfusion, Vol. 32, pgs. 529-533 (1992)) as well as in the remaining circulating platelets of the donor (Wun, et al., Transfusion, Vol. 32, pgs. 534-540 (1992)). These platelets may circulate for up to 48 hours after collection.

Activation of the platelets, however, eventually renders the platelets inactive and, therefore, in general, platelets, whether such platelets are contained in platelet concentrates or are single donor platelets, in general are discarded after 4 to 5 days.

It is an object of the present invention, therefore, to preserve platelets in vitro, thereby improving the quality of platelets used in transfusions.

In accordance with an aspect of the present invention, there is provided a method of preserving platelets. The method comprises contacting the platelets with an effective amount of nitric oxide.

Although the scope of the present invention is not intended to be limited to any theoretical reasoning, it is

believed that the addition of nitric oxide or a nitric oxide adduct inhibits, prevents, or retards the activation of platelets. The nitric oxide may inhibit the activation of platelets through increasing intracellular levels of cyclic GMP.

The treatment of the platelets with nitric oxide encompasses the use of gaseous nitric oxide and/or the use of a compound which is capable of delivering nitric oxide.

The term nitric oxide generally refers to the reactive forms of nitric oxide, in particular (1) uncharged nitric oxide ( $\text{NO}^\bullet$ ) (Gaseous nitric oxide is an uncharged form of nitric oxide); (2) negatively charged nitric oxide or  $\text{NO}^-$  (nitroxyl) and positively charged nitric oxide, or (3)  $\text{NO}^+$  (nitrosonium). Thus, the present invention contemplates the use of gaseous nitric oxide as well as compounds capable of donating or releasing nitric oxide in one of its reactive forms.

In one embodiment, the reactive form of nitric oxide is provided by gaseous nitric oxide.

In another embodiment, the reactive form of nitric oxide is provided by a compound which delivers nitric oxide. Compounds which deliver nitric oxide include, but are not limited to, S-nitrosothiols, S-nitroso amino acids, S-nitroso-polypeptides, and nitrosoamines.

Compounds contemplated for use in the invention are nitric oxide and compounds that release nitric oxide or otherwise directly or indirectly deliver or transfer nitric oxide to a site of its activity, such as on a cell membrane. As used herein, the term "nitric oxide" encompasses uncharged nitric oxide ( $\text{NO}^\bullet$ ) and charged nitric oxide species, particularly including nitrosonium ion ( $\text{NO}^+$ ) and nitroxyl ion ( $\text{NO}^-$ ). The nitric oxide releasing, delivering, or transferring compounds, having the structure  $\text{X-NO}$  wherein X is a nitric oxide releasing, delivering, or transferring moiety, include any and all such compounds which provide

nitric oxide to its intended site of action in a form active for their intended purpose. As used herein, the term "NO adducts" encompasses any of such nitric oxide releasing, delivering or transferring compounds. One group of such NO adducts is the S-nitrosothiols, which are compounds that include at least one -S-NO group. Such compounds include S-nitroso-polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); S-nitrosylated amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives thereof); S-nitrosated sugars, S-nitrosated-modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and an S-nitrosated hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; S-nitroso hydrocarbons having one or more substituent groups in addition to the S-nitroso group; and heterocyclic compounds. S-nitrosothiols and the methods for preparing them are described in U.S. Patent Application No. 07/943,834, filed September 14, 1992, Oae et al., Org. Prep. Proc. Int., 15(3):165-198 (1983); Loscalzo et al., J. Pharmacol. Exp. Ther., 249(3):726729 (1989) and Kowaluk et al., J. Pharmacol. Exp. Ther., 256:1256-1264 (1990), all of which are incorporated in their entirety by reference.

One particularly preferred embodiment of this aspect relates to S-nitroso amino acids where the nitroso group is linked to a sulfur group of a sulfur-containing amino acid or derivative thereof. For example, such compounds include the following: S-nitroso-N-acetylcysteine, S-nitroso-captopril, S-nitroso-homocysteine, S-nitroso-cysteine and S-nitroso-glutathione.

Suitable S-nitrosylated proteins include thiol-containing proteins (where the NO group is attached to one or

more sulfur groups on an amino acid or amino acid derivative thereof) from various functional classes including enzymes, such as tissue-type plasminogen activator (TPA) and cathepsin B; transport proteins, such as lipoproteins, heme proteins such as hemoglobin and serum albumin; and biologically protective proteins, such as the immunoglobulins and the cytokines. Such nitrosylated proteins are described in PCT Published Application No. WO 93/09806, published May 27, 1993.

Further examples of suitable S-nitrosothiols include those having the structures:



wherein x equals 2 to 20;

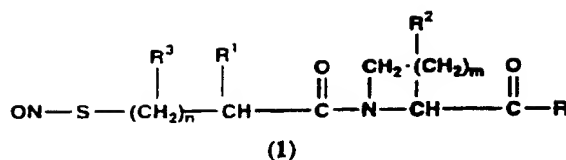


wherein x equals 2 to 20; and



wherein x equals 2 to 20 and Y is selected from the group consisting of fluoro,  $\text{C}_1$ - $\text{C}_6$  alkoxy, cyano, carboxamido,  $\text{C}_3$ - $\text{C}_6$  cycloalkyl, aralkoxy,  $\text{C}_2$ - $\text{C}_6$  alkylsulfinyl, arylthio,  $\text{C}_1$ - $\text{C}_6$  alkylamino,  $\text{C}_2$ - $\text{C}_{15}$  dialkylamino, hydroxy, carbamoyl,  $\text{C}_1$ - $\text{C}_6$  N-alkylcarbamoyl,  $\text{C}_2$ - $\text{C}_{15}$  N,N-dialkylcarbamoyl, amino, hydroxyl, carboxyl, hydrogen, nitro and aryl; wherein aryl includes benzyl, naphthyl, and anthracenyl groups.

Other suitable S-nitrosothiols that are S-nitroso-angiotensin converting enzyme inhibitors (hereinafter referred to as S-nitroso-ACE inhibitors) are described in Loscalzo, U.S. Patent No. 5,002,964 (1991) and Loscalzo et al., U.S. Patent No. 5,025,001 (1991) both of which are incorporated in their entirety by reference. Examples of such S-nitroso-ACE inhibitors include compounds having structure (1):



wherein

R is hydroxy,  $\text{NH}_2$ ,  $\text{NHR}^4$ ,  $\text{NR}^4\text{R}^5$ , or  $\text{C}_1$ - $\text{C}_7$  alkoxy, wherein  $\text{R}^4$  and  $\text{R}^5$  are  $\text{C}_1$ - $\text{C}_4$  alkyl, or phenyl, or  $\text{C}_1$ - $\text{C}_4$  alkyl substituted by phenyl;

$\text{R}^1$  is hydrogen,  $\text{C}_1$ - $\text{C}_7$  alkyl, or  $\text{C}_1$ - $\text{C}_7$  alkyl substituted by phenyl, amino, guanidino,  $\text{NHR}^6$ ,  $\text{NR}^6\text{R}^7$ , wherein  $\text{R}^6$  and  $\text{R}^7$  are methyl or  $\text{C}_1$ - $\text{C}_4$  alkanoyl;

$\text{R}^2$  is hydrogen, hydroxy,  $\text{C}_1$ - $\text{C}_4$  alkoxy, phenoxy, or  $\text{C}_1$ - $\text{C}_7$  alkyl;

$\text{R}^3$  is hydrogen,  $\text{C}_1$ - $\text{C}_4$  or  $\text{C}_1$ - $\text{C}_7$  alkyl substituted by phenyl;

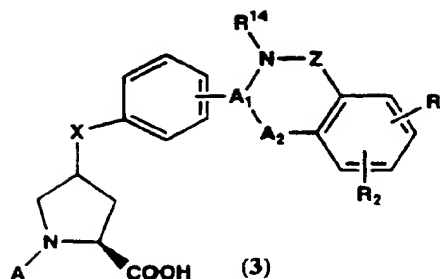
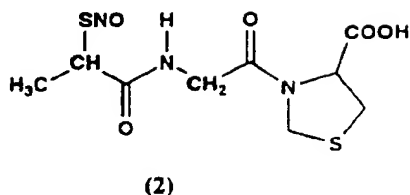
m is 1 to 3; and

n is 0 to 2.

Other suitable S-nitroso-ACE inhibitors include N-acetyl-S-nitroso-D-cysteinyl-L-proline, N-acetyl-S-nitroso-D, L-cysteinyl-L-proline, 1-(4-amino-2-S-nitroso)mercaptomethylbutanoyl-L-proline, 1-[2-hexanoyl]-L-proline, 1-[5-guanidino-2-(S-nitroso)mercaptomethyl-pentanoyl]-L-proline, 1-[5-amino-2-(S-nitroso)mercaptomethyl-pentanoyl]-4-hydroxy-L-proline, 1-[5-guanidino-2-(S-nitroso)mercaptomethyl-pentanoyl]-4-hydroxy-L-proline, 1-[2-aminomethyl-3(S-nitroso)-mercaptomethyl-pentanoyl]-L-proline, and S-nitroso-L-cysteinyl-L-proline.

Additional suitable S-nitroso-ACE inhibitors include those having structures (2-3):





wherein

X is oxygen or sulfur;

$-A_1, -A_2-$  is  $CH-NH$  or  $-C=N-$ ;

A is  $ON-S-CH_2-CH-C(=O)-R_3$ ;

R is selected from hydrogen, lower ( $C_1-C_4$ ) alkyl, benzyl, benzhydryl, and salt forming ion;

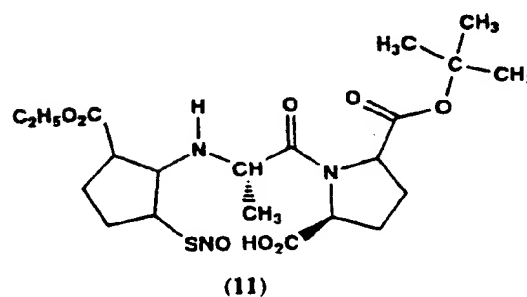
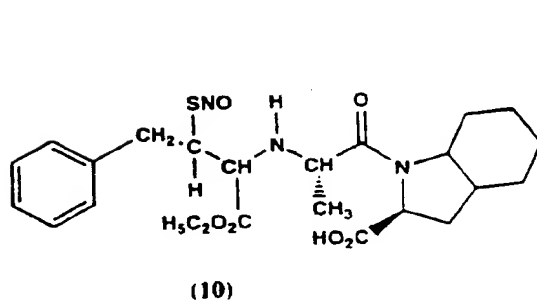
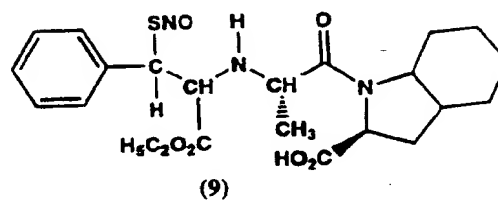
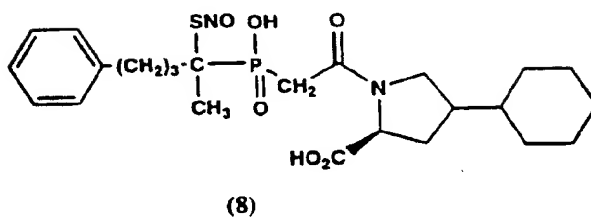
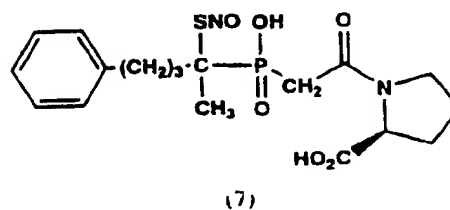
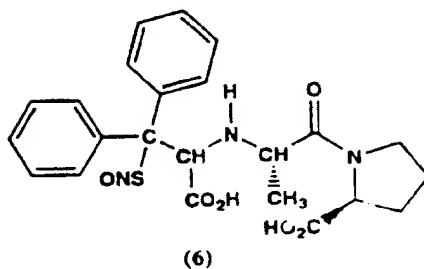
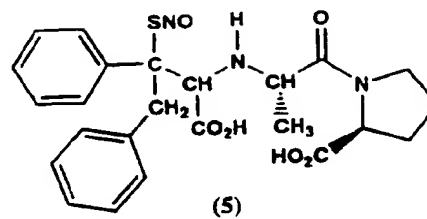
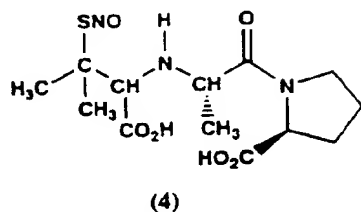
$R_1$  and  $R_2$  are independently selected from hydrogen, halogen, lower alkyl, lower alkoxy, halo substituted lower alkyl, nitro, and  $SO_2NH_2$ ;

Z is  $-C(=O)-$  or  $-S(=O)_2-$

$R_3$  is hydrogen, lower alkyl, halo substituted lower alkyl, phenyl, benzyl, phenethyl, or cycloalkyl; and

$R_4$  is hydrogen, lower alkyl, halo substituted lower alkyl, hydroxy substituted lower alkyl,  $-(CH_2)_q-N$  (lower alkyl)<sub>2</sub> or  $-(CH_2)_q-NH_2$  and q is one, two, three or four.

Additional suitable compounds include those having structures (4-11):



The S-nitroso-ACE inhibitors can be prepared by various methods of synthesis. In general, the thiol precursor is

prepared first, then converted to the S-nitrosothiol derivative by nitrosation of the thiol group with  $\text{NaNO}_2$  under acidic conditions ( $\text{pH} = 1$  to  $5$ ) which yields the S-nitroso derivative. Acids which may be used for this purpose include aqueous sulfuric, acetic and hydrochloric acids. Thiol precursors are prepared as described in the following: U.S. Pat. Nos. 4,046,889 (1977); 4,052,511; 4,053,651; 4,113,751, 4,154,840, 4,129,571 (1978), and 4,154,960 (1979) to Ondetti et al.; U.S. Pat. No. 4,626,545 (1986) to Taub; and U.S. Pat. Nos. 4,692,458 (1987) and 4,692,459 (1987) to Ryan et al., Quadro, U.S. Pat. No. 4,447,419 (1984); Haugwitz et al.; U.S. Pat. No. 4,681,886 (1987), Bush et al., U.S. Pat. No. 4,568,675 (1986), Bennion et al., U.S. Pat. No. 4,748,160 (1988), Portlock, U.S. Pat. No. 4,461,896 (1984), Hoefle et al., European Patent Application Publication No. 0 088 341 (1983), Huange et al., U.S. Pat. No. 4,585,758 (1986), European Patent application Publication No. 0 237 239, European Patent application Publication No. 0 174 162, published in 1986, European Patent application Publication No. 0 257 485, published in 1988, all of which are incorporated by reference herein.

Another group of such NO adducts are compounds that include at least one -O-NO group. Such compounds include O-nitroso-polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); O-nitrosylated amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives thereof); O-nitrosated sugars; O-nitrosated-modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and an O-nitrosated hydrocarbon where the hydrocarbon can be a branched or unbranched, saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; O-nitroso hydrocarbons having one

or more substituent groups in addition to the O-nitroso group; and heterocyclic compounds.

Another group of such NO adducts is the nitrites which have an -O-NO group wherein R is a protein, polypeptide, amino acid, branched or unbranched and saturated or unsaturated alkyl, aryl or a heterocyclic. A preferred example is the nitosylated form of isosorbide. Compounds in this group form S-nitrosothiol intermediates *in vivo* in the recipient human or other animal to be treated and can therefore include any structurally analogous precursor R-O-NO of the S-nitrosothiols described above.

Another group of such NO adducts is the N-nitrosoamines, which are compounds that include at least one -N-NO group. Such compounds include N-nitroso-polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); N-nitrosylated amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures); N-nitrosated sugars; N-nitrosated-modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and an N-nitrosated hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; N-nitroso hydrocarbons having one or more substituent groups in addition to the N-nitroso group; and heterocyclic compounds.

Another group of such NO adducts is the C-nitroso compounds that include at least one -C-NO group. Such compounds include C-nitroso-polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); C-nitrosylated amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures); C-nitrosated sugars; C-nitrosated-modified

and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and a C-nitrosated hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; C-nitroso hydrocarbons having one or more substituent groups in addition to the C-nitroso group; and heterocyclic compounds.

Another group of such NO adducts is the nitrates which have at least one -O-NO<sub>2</sub> group. Such compounds include polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives thereof); sugars; modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and a hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; hydrocarbons having one or more substituent groups; and heterocyclic compounds. A preferred example is nitroglycerin.

Another group of such NO adducts is the nitroso-metal compounds which have the structure (R)<sub>n</sub>-A-M-(NO)<sub>x</sub>. R includes polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives thereof); sugars; modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and a hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon;

hydrocarbons having one or more substituent groups in addition to the A-nitroso group; and heterocyclic compounds.

A is S, O, or N, n and x are each integers independently selected from 1, 2 and 3, and M is a metal, preferably a transition metal. Preferred metals include iron, copper, manganese, cobalt, selenium and ruthenium. Also contemplated are N-nitrosylated metal centers such as nitroprusside.

Another group of such NO adducts is the N-oxo-N-nitrosoamines which have an  $R-N(O^-M^+)-NO$  group or an  $R-NO-NO$ -group. R includes polypeptides (the term "polypeptide" includes proteins and also polyamino acids that do not possess an ascertained biological function, and derivatives thereof); amino acids (including natural and synthetic amino acids and their stereoisomers and racemic mixtures and derivatives thereof); sugars; modified and unmodified oligonucleotides (preferably of at least 5, and more particularly 5-200, nucleotides); and a hydrocarbon where the hydrocarbon can be a branched or unbranched, and saturated or unsaturated aliphatic hydrocarbon, or an aromatic hydrocarbon; hydrocarbons having one or more substituent groups; and heterocyclic compounds. R is preferably a nucleophilic (basic) moiety.  $M^+$  is a metal cation, such as, for example, a Group I metal cation.

Another group of such NO adducts is the thionitrates which have the structure  $R-(S)_x-NO$  wherein x is an integer of at least 2. R is as described above for the S-nitrosothiols. Preferred are the dithiols wherein x is 2. Particularly preferred are those compounds where R is a polypeptide or hydrocarbon and a pair or pairs of thiols are sufficiently structurally proximate, i.e. vicinal, that the pair of thiols will be reduced to a disulfide. Those compounds which form disulfide species release nitroxyl ion ( $NO^-$ ) and uncharged nitric oxide ( $NO^\bullet$ ). Those compounds where the thiol groups are not sufficiently close to form disulfide bridges generally

only provide nitric oxide as the  $\text{NO}^-$  form but not as the uncharged  $\text{NO}^\bullet$  form.

The platelets are contacted with the compound (gaseous nitric oxide or a nitric oxide adduct) in an amount effective to inhibit or prevent platelet activation or aggregation. In one embodiment, the platelets are contacted with the compound in an amount of from about 10 nM to about 10 mM, preferably from about 10 nM to about 1 mM. The compound may be added to platelets contained in platelet concentrates or to single donor platelets. The compounds may be added to the platelets at any stage of the processing of the platelets, such as (i) during their collection via whole blood donation or apheresis; or (ii) during manipulations of whole blood or fractions thereof performed in the course of isolating, concentrating, or washing platelets; or (iii) during storage of platelets, whether at room temperature, in a refrigerator, or in a freezer. In a particularly preferred embodiment, the treatment is effected prior to treatment of the platelet concentrates.

It is also understood that, within the scope of the present invention, an anticoagulant or anti-thrombogenic agent may be added to the platelets in combination with the nitric oxide or nitric oxide adduct. The terms "anticoagulant" and "anti-thrombogenic agent" as used herein mean any compound which alters platelet function or interferes with other mechanisms involved in blood clotting. Examples of such compounds include, but are not limited to, heparin, warfarin, aspirin, indomethacin, dipyridamole, sulfinpyrazone, and other non-steroidal anti-inflammatory drugs.

The nitric oxide or nitric-oxide adduct may be added to the platelets in combination with a physiologically acceptable carrier. Examples of such physiologically acceptable carriers include, but are not limited to, water, and saline solutions. Such a combination also can be

sterilized and may be mixed with auxiliary agents such as preservatives, stabilizers, wetting agents, salts or buffers which do not react deleteriously with the nitric oxide or nitric oxide adduct, or with the platelets, or with other blood components.

In accordance with another aspect of the present invention, there is provided a method of preserving platelets comprising contacting platelets with an effective amount of cyclic GMP or a derivative or analogue thereof. The cyclic GMP may be administered alone or in combination with the gaseous nitric oxide or nitric oxide adduct hereinabove described.

Examples of cyclic GMP (cGMP) derivatives or analogues thereof which may be employed include, but are not limited to, dibutyl cGMP, 8-bromo-cGMP, 8-(p-chlorophenylthio)-cGMP (8-pCPT-cGMP), and  $\beta$ -phenyl-1, N-etheno-cGMP (1, N-PET-cGMP). The cyclic GMP or derivative or analogue thereof may be added to the platelets at any stage during processing of the platelets, as hereinabove described with respect to nitric oxide or nitric oxide adducts. In one embodiment, the platelets are contacted with the cGMP or derivative or analogue thereof in an amount of from about 10 nM to about 10 mM, preferably from about 10 nM to about 1 mM.

The invention now will be described with respect to the following examples; however, the scope of the present invention is not intended to be limited thereby.

#### Example 1

Whole blood was collected in CPD anticoagulant in a blood donor center. Two centrifugation steps then were used to prepare platelet concentrates. In the first step, whole blood (450 ml  $\pm$  45 ml) was subjected to a light spin (2000xg for 5 minutes), after which the resulting supernatant fluid (platelet rich plasma or PRP) was removed. 100  $\mu$ M of nitric oxide, or S-nitroso-N-acetylcysteine, or S-nitrosoglutathione



or an equivalent amount of saline (control) was added to the bags of platelet-rich plasma samples. The control sample received no nitric oxide or S-nitrosothiols. Two samples were treated with nitric oxide, one sample was treated with S-nitroso-N-acetylcysteine, one sample was treated with S-nitroso-glutathione, and one sample was a control sample.

The platelet-rich plasma then was subjected to a "heavy spin" (5000xg for 5 minutes), which pellets the platelets. Plasma then was expressed until 30 to 50 ml remained. The platelet bags were left stationary for 1 hour at room temperature. The platelets then were resuspended by gentle manual manipulation of the bag and placed on a rotator for storage. Activation of platelets was measured by expression of P-selectin (GMP-140 or CD62) using flow cytometry for 120 hours. The results are shown in Figure 1.

As shown in Figure 1, platelet activation was significantly lower in those samples treated with nitric oxide, S-NO-acetylcysteine, or S-NO-glutathione, as compared with the control (untreated) sample.

#### Example 2

Platelet-rich plasma was prepared as described in Example 1. NO donors in the form of S-nitroso-N-acetylcysteine or S-nitrosoglutathione or nitric oxide are added in an amount of 100  $\mu$ M to 8 of the samples of the platelet-rich plasma. The samples were subjected to a heavy spin, and platelet concentrates were prepared as described in Example 1. Thirteen (13) untreated samples served as controls. After the control and treated platelet samples have been incubated for 1 hour at room temperature, samples were drawn for testing for expression of P-selectin (GMP-140 or CD62) as described hereinabove in Example 1. After the platelet samples (8 treated samples and 13 controls) have been stored for 5 days, the platelets were assayed for expression of P-selectin (GMP-140 or CD62), also as

hereinabove described, whereby the percent of activated platelets was measured. The average percent platelet activation for treated and untreated platelets at 1 hour and 5 days of storage is shown in Figure 2.

As shown in Figure 2, the platelet samples treated with NO donors exhibited a lower percentage of platelet activation at both 1 hour and 5 days after storage, as compared with the untreated control samples.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. A method of preserving platelets, comprising:  
contacting platelets with an effective platelet preserving amount of nitric oxide.
2. The method of Claim 1 wherein said platelets are contacted with said compound at a concentration of from about 10 nM to about 10 mM.
3. The method of Claim 2 wherein said platelets are contacted with said compound at a concentration of from about 10 nM to about 1 mM.
4. The method of claim 1 wherein the treating is effected by administering gaseous nitric oxide or a compound which delivers nitric oxide.
5. The method of claim 4 wherein the compound is selected from the group consisting of S-nitrosothiols, compounds that include at least one -O-NO group, N-nitrosoamines, C-nitroso compounds including at least one -C-NO group, nitrates having at least one -O-NO<sub>2</sub> group, nitroso-metal compounds, N-oxo-N-nitrosoamines, and thionitrates.
6. The method of claim 5 wherein the compound is an S-nitrosothiol.
7. The method of Claim 6 wherein said S-nitrosothiol is an S-nitroso-amino acid.
8. The method of Claim 6 wherein said S-nitrosothiol is an S-nitrosylated protein.
9. The method of Claim 6 wherein said S-nitrosothiol is an S-nitroso-polypeptide.
10. The method of claim 6 wherein the S-nitrosothiol is selected from the group consisting of those having the structures:



wherein x equals 2 to 20;

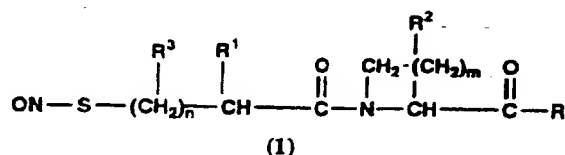


wherein x equals 2 to 20; and



wherein x equals 2 to 20 and Y is selected from the group consisting of fluoro, C<sub>1</sub>-C<sub>6</sub> alkoxy, cyano, carboxamido, C<sub>3</sub>-C<sub>6</sub> cycloalkyl, aralkoxy, C<sub>2</sub>-C<sub>6</sub> alkylsulfinyl, arylthio, C<sub>1</sub>-C<sub>6</sub> alkylamino, C<sub>2</sub>-C<sub>15</sub> dialkylamino, hydroxy, carbamoyl, C<sub>1</sub>-C<sub>6</sub> N-alkylcarbamoyl, C<sub>2</sub>-C<sub>15</sub> N,N-dialkylcarbamoyl, amino, hydroxyl, carboxyl, hydrogen, nitro and aryl; wherein aryl includes benzyl, naphthyl, and anthracenyl groups.

11. The method of claim 6 wherein the S-nitrosothiol is an S-nitroso-ACE inhibitor selected from the group consisting of compounds having the following structure (1):



wherein

R is hydroxy, NH<sub>2</sub>, NHR<sup>4</sup>, NR<sup>4</sup>R<sup>5</sup>, or C<sub>1</sub>-C<sub>7</sub> alkoxy, wherein R<sup>4</sup> and R<sup>5</sup> are C<sub>1</sub>-C<sub>4</sub> alkyl, or phenyl, or C<sub>1</sub>-C<sub>4</sub> alkyl substituted by phenyl;

R<sup>1</sup> is hydrogen, C<sub>1</sub>-C<sub>7</sub> alkyl, or C<sub>1</sub>-C<sub>7</sub> alkyl substituted by phenyl, amino, guanidino, NHR<sup>6</sup>, NR<sup>6</sup>R<sup>7</sup>, wherein R<sup>6</sup> and R<sup>7</sup> are methyl or C<sub>1</sub>-C<sub>4</sub> alkanoyl;

R<sup>2</sup> is hydrogen, hydroxy, C<sub>1</sub>-C<sub>4</sub> alkoxy, phenoxy, or C<sub>1</sub>-C<sub>7</sub> alkyl;

R<sup>3</sup> is hydrogen, C<sub>1</sub>-C<sub>4</sub> or C<sub>1</sub>-C<sub>7</sub> alkyl substituted by phenyl;

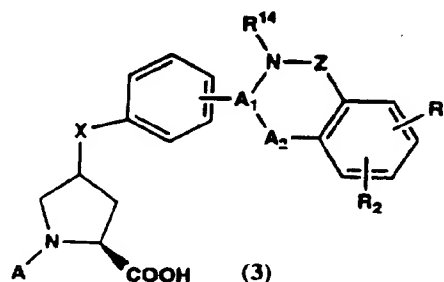
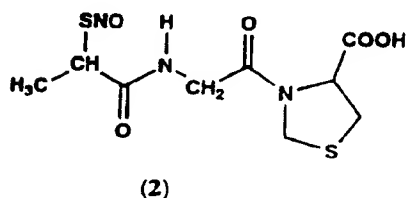
m is 1 to 3; and

n is 0 to 2.

12. The method of claim 6 wherein the S-nitrosothiol is an S-nitroso-ACE inhibitor selected from the group consisting of N-acetyl-S-nitroso-D-cysteinyl-L-proline, N-acetyl-S-nitroso-D,L-cysteinyl-L-proline, 1-(4-amino-

2-S-nitroso)mercaptomethyl butanoyl)-L-proline, 1-[2-hexanoyl]-L-proline, 1-[5-guanidino-2-(S-nitroso)mercaptomethyl-pentanoyl]-L-proline, 1-[5-amino-2-(S-nitroso)mercaptomethyl-pentanoyl]-4-hydroxy-L-proline, 1-[5-guanidino-2-(S-nitroso)mercaptomethyl-pentanoyl]-4-hydroxy-L-proline, 1-[2-aminomethyl-3(S-nitroso)-mercaptomethyl-pentanoyl]-L-proline, and S-nitroso-L-cysteiny-L-proline.

13. The method of claim 6 wherein the S-nitrosothiol is an S-nitroso-ACE inhibitor selected from the group consisting of compounds having structures (2-3):



wherein

X is oxygen or sulfur;

-A<sub>1</sub>, -A<sub>2</sub>- is CH-NH or -C=N-;

A is  $\text{ON-S-CH}_2\text{-CH-C(=O)R}_3$ ;

R is selected from hydrogen, lower (C<sub>1</sub>-C<sub>4</sub>) alkyl, benzyl, benzhydryl, and salt forming ion;

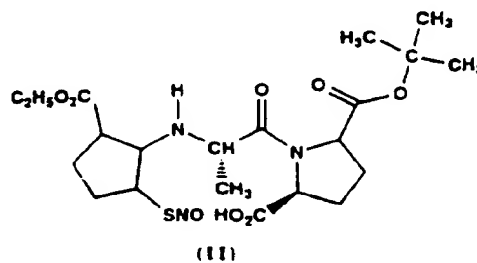
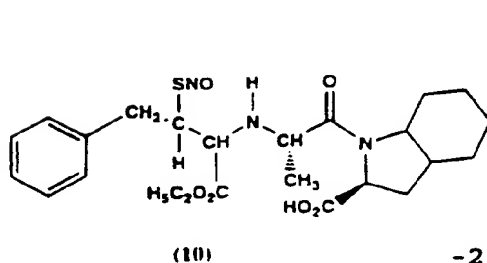
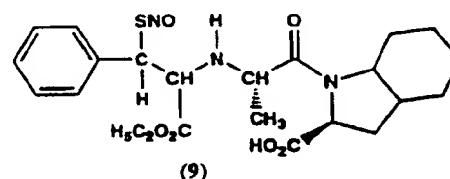
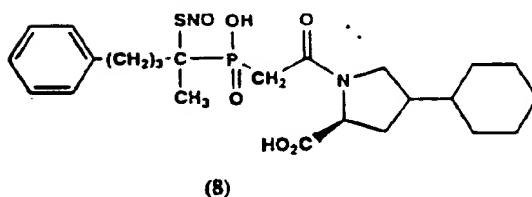
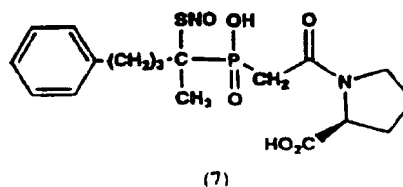
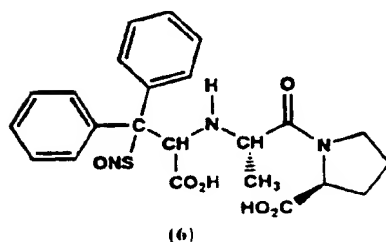
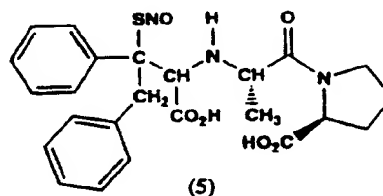
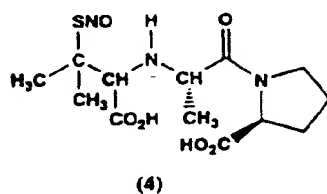
R<sub>1</sub> and R<sub>2</sub> are independently selected from hydrogen, halogen, lower alkyl, lower alkoxy, halo substituted lower alkyl, nitro, and SO<sub>2</sub>NH<sub>2</sub>;

Z is  $\text{-C(=O)-}$  or  $\text{-S(=O)}_2\text{-}$

$R_3$  is hydrogen, lower alkyl, halo substituted lower alkyl, phenyl, benzyl, phenethyl, or cycloalkyl; and

$R_4$  is hydrogen, lower alkyl, halo substituted lower alkyl, hydroxy substituted lower alkyl,  $-(CH_2)_q-N$  (lower alkyl)<sub>2</sub> or  $-(CH_2)_q-NH_2$  and  $q$  is one, two, three or four.

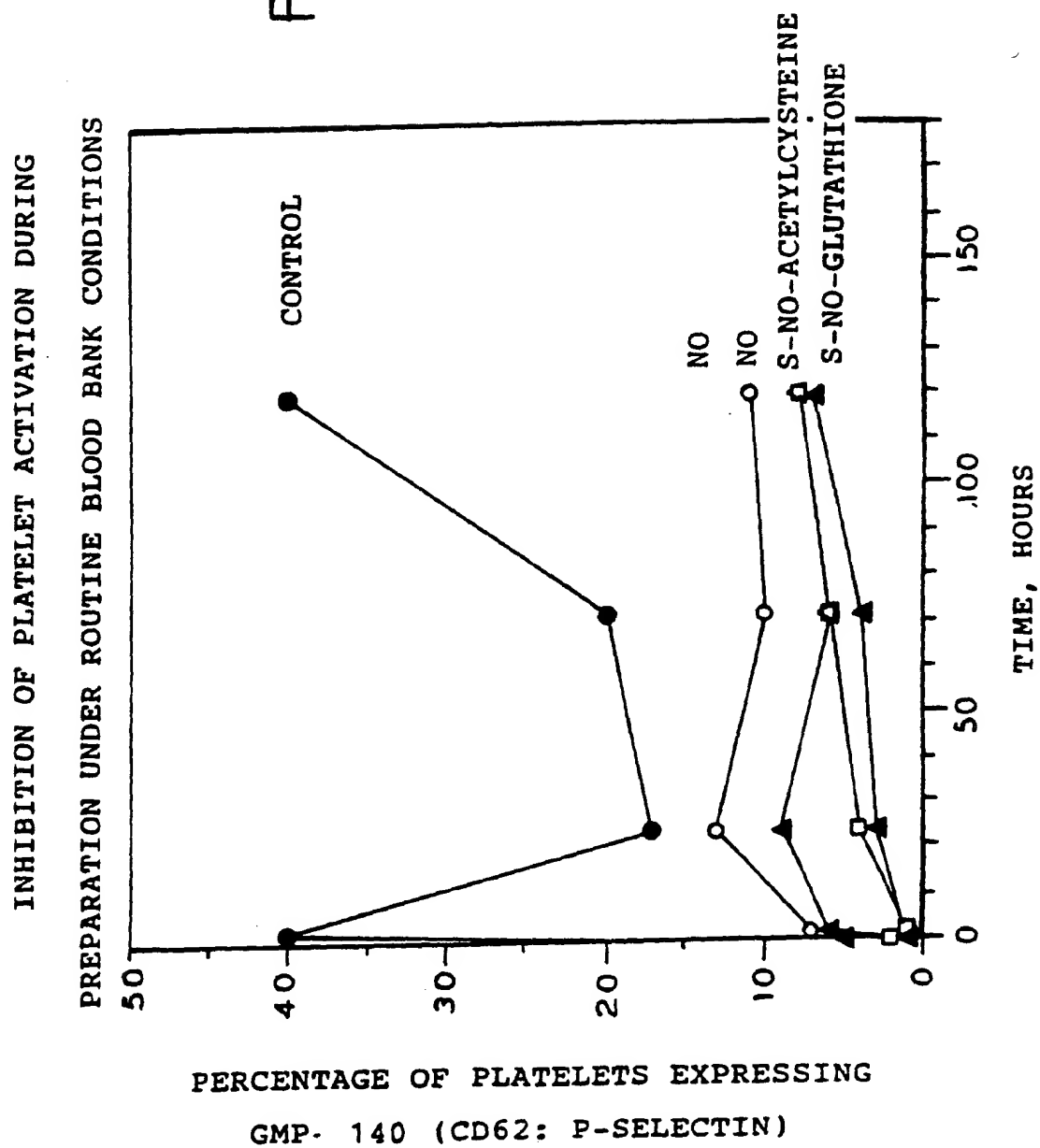
14. The method of claim 6 wherein the S-nitrosothiol is an S nitroso-ACE inhibitor selected from the group consisting of compounds having structures (4-11):



15. A method of preserving platelets comprising:  
contacting platelets with an effective amount of cyclic GMP or an analogue or derivative thereof.
16. The method of Claim 15 wherein said platelets are contacted with said cyclic GMP or an analogue or derivative thereof at a concentration of from about 10 nM to about 10 mM.
17. The method of Claim 16 wherein said platelets are contacted with said cyclic GMP or an analogue or derivative thereof at a concentration of from about 10 nM to about 1 mM.

1 / 2

FIG. 1

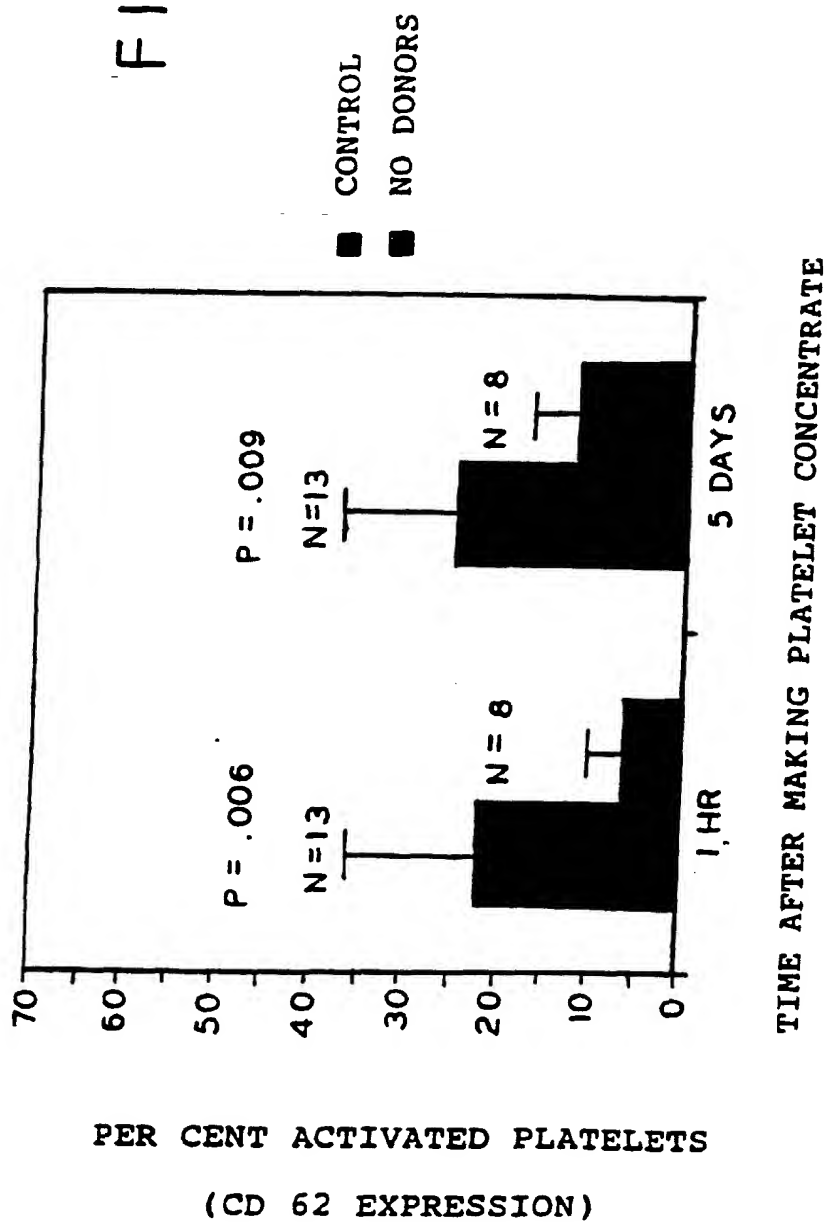


SUBSTITUTE SHEET (RULE 26)



2 / 2

EFFECT OF NO DONORS UPON PLATELET ACTIVATION DURING  
PLATELET CONCENTRATE PREPARATION IN A BLOOD BANK



SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09287

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00, 38/16, 31/79, 31/195, 31/13, 31/16  
US CL : 514/2, 6, 20, 557, 562, 563, 611, 613, 616, 625, 626, 629  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 6, 20, 557, 562, 563, 611, 613, 616, 625, 626, 629

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Circulation Research, Volume 68, Number 6, issued June 1991, Lieberman et al., "S-Nitrosocysteine Inhibition of Human Platelet Secretion Is Correlated With Increases in Platelet cGMP Levels", pages 1722-1728, see entire document.	1-6
X	Thrombosis Research, Volume 50, issued 1988, Radomski et al., "Isolation and Washing of Human Platelets with Nitric Oxide", pages 537-546, see entire document.	1-4
X	British Journal of Pharmacology, Volume 107, issued 1992, Radomski et al., "S-nitro-glutathione inhibits platelet activation invitro and in vivo", pages 745-749, see entire document.	1-7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 OCTOBER 1995

Date of mailing of the international search report

27 NOV 1995

Name and mailing address of the ISA/US  
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09287

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/09287

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-14, drawn to preserving platelets using nitric oxide.

Group II, claim(s) 15-17, drawn to preserving platelets using cyclic GMP.

The inventions listed as Groups I & II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the inventive concept for group I is considered to be utilizing nitric oxide to preserve platelets and Group II utilizes a completely different component.